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Intra- and interspecific phenotypic characteristics of fish-pathogenic *Edwardsiella ictaluri* and *E. tarda*

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Abstract

Intra- and interspecific characteristics of fish-pathogenic *Edwardsiella ictaluri*, and *E. tarda* were determined by numerical analysis of gel electrophoresed protein profiles, fatty acid methyl esters (FAMEs) and immunoblotting. The 18 *E. ictaluri* isolates revealed a high degree of homogeneity (70% similarity or higher) in their protein profiles and 95% similarity in their FAME, while the nine *E. tarda* isolates revealed 30% similarity in their protein profiles and 95% similarity in their FAME. Immunoblots probed for antigenic epitopes with goat antiserum produced against *E. ictaluri* and *E. tarda*, respectively, revealed that *E. ictaluri* were more homogeneous compared with the *E. tarda* isolates. Overall, there was a considerable degree of relatedness between the two species. Our findings suggest that phenotypically *E. ictaluri* represents a clonal bacterial population structure compared with the less monomorphic *E. tarda*.

Keywords: *Edwardsiella*, catfish, proteins, fatty acids, antigens

Introduction

Edwardsiella ictaluri and *E. tarda* are facultative aerobic, Gram-negative bacteria that belong to the family *Enterobacteriaceae*. Both species of *Edwardsiella* are important pathogens that produce enteroheamorrhagic septicaemic disease in a variety of commercially raised food fish. While *E. ictaluri* typically produces enteric septicaemia in catfish (*Ictalurus punctatus* Rafinesque) and infrequently in other species thriving in freshwater habitats (Plumb and Sanchez 1983; Plumb 1999), *E. tarda* has a broader host range infecting both freshwater and marine fish species as well as several terrestrial animals including man (Willson, Waterer, Wofford Jr & Chapman 1989; Janda, Abbott, Kroske-Bystrom, Cheung, Powers, Kokka & Tamura 1991). Disease caused by *E. ictaluri* and *E. tarda*, has a significant economic impact on the aquaculture industry in countries where fish farming constitutes a major agricultural enterprise.

On account of the occurrence of disease in geographically widespread regions and the broad host range, it has been of interest to determine whether *Edwardsiella* isolates originating from disparate outbreaks of disease in fish exhibit uniquely distinguishable phenotypic characteristics. Morphologically, both species *E. ictaluri* and *E. tarda* are indistinguishable. However, they possess few distinctive biochemical characteristics that set them apart (Holmes, Dawson & Pinning 1986; Farmer 2003). Serological typing has been attempted using a variety of methods in a quest to determine specific antigenic differences within isolates belonging to each of the species. Rogers (1981) reported positive reactions with 20 *E. ictaluri* isolates examined with rabbit antiserum produced against the type-strain of *E. ictaluri* (ATCC-33202). Bertolini, Cipriano, Pyle and McLaughlin (1990) examined the serological relationship among 32 isolates of *E. ictaluri* and concluded that most isolates of *E. ictaluri* belong to a single antigenic serotype. Using a panel of monoclonal antibodies to examine 18 *E. ictaluri* isolates with the fluorescent antibody technique, Plumb and Klesius (1988) found antigenic variations among isolates but consistency in the recognition of two major epitopes of 34 and 60 kDa molecular mass among all isolates by

immune sera from channel catfish. Analysis of outer membrane protein profiles of 33 *E. ictaluri* isolates by gel electrophoresis has shown that with a few exceptions, most isolates revealed a uniform pattern (Newton, Blevins, Wilt & Wolfe 1990). Other studies have shown differences between *E. ictaluri* isolates on the basis of red blood cell agglutination–inhibition by the monosaccharide D-mannose (Wong, Miller & Janda 1989; Ainsworth 1993) and in challenge studies with heterologous isolates of *E. ictaluri* on immunized catfish (Klesius & Shoemaker 1997).

While the evidence for homogeneity among isolates within the species of *E. ictaluri* remains rather inconclusive, there is some evidence supporting the phenotypic diversity present among isolates of *E. tarda*. Based on the somatic (O) and flagella (H) antigens of *E. tarda*, a number of serovar groups have been identified within this species (Sakazaki 1984; Tamura, Sakazaki, McWhorter & Kosako 1988). Following the discovery of two proteins, a flagellin and SseB a protein belonging to the type III secretion system, a nominal distinction between virulent and avirulent *E. tarda* has been made by comparative proteomic analysis of 14 isolates (Tan, Lin, Wang, Joshi, Hew & Leung 2002). Random amplified polymorphic DNA profiles of *E. tarda* isolated from fish and humans from diverse geographic locations have also shown recognizable differences among isolates (Nucci, da Silveira, da Silva Correa, Nakazato, Bando, Ribeiro & Pestana de Coatro 2002). Succinctly, the studies reviewed may reflect that among isolates of *E. ictaluri* there is appreciable homogeneity, while the closely related species *E. tarda* displays varying degrees of heterogeneity. However, the evidence is not overbearing and underscores the need for further analysis of *Edwardsiella* isolates. The present study is focused on substantiating with additional evidence the claims for homogeneity and/or diversity that exist among isolates of *E. ictaluri* and *E. tarda* from different geographic locations on the basis of several recognized phenotypic criteria.

Materials and methods

Bacteria and culture conditions

The 27 isolates of *Edwardsiella* used together with their reference numbers, their source and other descriptive information are listed in Table 1. For biochemical characterization and for cellular fatty acid analysis, bacteria were cultured on blood agar (tryptic soy agar with 5% defibrinated sheep blood)

Table 1 *Edwardsiella icataluri* and *E. tarda* isolates used in this study

Sample #	Species	Isolate	Source	Origin
1.	*E. ictaluri*	016-S99-1911*	Catfish	Mississippi
2.	*E. ictaluri*	017-S99-1914	Catfish	Mississippi
3.	*E. ictaluri*	013-S99-1908	Catfish	Mississippi
4.	*E. ictaluri*	003-S99-1760	Catfish	Mississippi
5.	*E. ictaluri*	S-94-1051	Catfish	Mississippi
6.	*E. ictaluri*	ALG-03-189†	Catfish	Alabama
7.	*E. ictaluri*	ALG-03-190	Catfish	Alabama
8.	*E. ictaluri*	ALG-99-407	Catfish	Alabama
9.	*E. ictaluri*	ALG-03-192	Catfish	Alabama
10.	*E. ictaluri*	ALG-03-161	Catfish	Alabama
11.	*E. ictaluri*	AL-93-75‡	Catfish	Alabama
12.	*E. ictaluri*	AL-93-58	Catfish	Alabama
13.	*E. ictaluri*	AL-95-73	Catfish	Alabama
14.	*E. ictaluri*	RE-33§	Catfish	Alabama
15.	*E. ictaluri*	E-18	Catfish	Mississippi
16.	*E. ictaluri*	EILO¶	Catfish	Alabama
17.	*E. ictaluri*	IA-30-NJ#1‖	Tadpole madtom, *Noturus gyrinus* (Mitchill)	New Jersey
18.	*E. ictaluri*	ATCC-33202**	Catfish	ATCC (type strain)
19.	*E. tarda*	AU-TN-03	Catfish	Alabama
20.	*E. tarda*	AL-HSB-K-03	Catfish	Alabama
21.	*E. tarda*	AL-03-32	Bluegill, *Lepomis macrochirus* (Rafinesque)	Alabama
22.	*E. tarda*	AL-98-87	Catfish	Alabama
23.	*E. tarda*	AL-97-052	Catfish	Alabama
24.	*E. tarda*	ATCC-15947	Human	ATCC (type strain)
25.	*E. tarda*	AL-92-255	Largemouth Bass, *Micropterus salmoide* (Lacepede)	Alabama
26.	*E. tarda*	AL-97-38	Tilapia, *Oreochromis Niloticus* (Linnaeus)	Alabama
27.	*E. tarda*	FL-95-01	Catfish	Florida

*Isolated from diseased catfish in Mississippi. Dr David Wise, Fish Diagnostic Laboratory, Thad Cochran National Warmwater Aquaculture Center, Stoneville, MS, USA.

†All isolates with the prefix ALG were isolated from diseased catfish in Alabama. Mr. William Hemstreet, Alabama Fish Farming Center, Greensboro, AL, USA.

‡All isolates with the prefix AL/AU were isolated from diseased fish in Alabama by the Auburn University Fish Diagnostic Laboratory, Auburn University, AL, USA.

§Avirulent vaccine strain used in commercial vaccine, derived from EILO; Klesius and Shoemaker (1997).

¶Kasornchandra, Rogers and Plumb. (1987).

‖Klesius et al. (2003).

**Hawke, McWhorter, Steigerwalt and Brenner (1981).

plates at 28 °C. For analysis of electrophoretic protein profiles, bacteria were initially cultured on brain heart infusion (BHI) agar and single colonies were picked and cultured in BHI broth at 28 °C in a shaker water bath. Logarithmic-phase bacteria ($2–4 \times 10^8$ CFU mL^{-1}) were used in the later tests.

Biochemical tests

The identity of the reference strains for each respective species *E. ictaluri* (ATCC-33202) and *E. tarda* (ATCC-15947) was confirmed by conventional biochemical tests using the identification card (ID-GNI) for Gram-negative bacilli with the VITEK 32 system (bioMerieux Vitek, Hazelwood, MO, USA) and the API 20E test strips (bioMerieux) following the instructions provided. The results were compared with probability matrix for identification of Gram-negative aerobic fermentative bacteria (Holmes *et al.* 1986) and the biochemical reaction profiles for identification of enteric groups in the family *Enerobacteriaceae* (Farmer 2003). All isolates used in this study were similarly screened using the VITEK 32 system and the API 20E test strips. Some isolates were randomly tested more than twice.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Bacteria were pelleted by centrifugation at 8000 *g* for 15 min, washed three times in tris-buffered saline (25 mM Tris-HCl, 0.15 M NaCl, pH 7.2) and resuspended in Laemmli (1970) sample buffer to yield a protein concentration of $\sim$ 830 μg mL^{-1}, determined with the bicinchroninic acid (BCA) protein assay (Pierce Biotechnology, Rockford, IL, USA). Thirty microlitres of each sample was loaded onto the gel and SDS-PAGE was conducted essentially according to the method of Laemmli (1970) using Criterion (Bio-Rad, Hercules, CA, USA) precast, 10–20% linear gradient slab gels. Electrophoresis was at 175 V (constant voltage) at 15 °C for $\sim$ 90 min. Duplicate gels were run simultaneously and one of the gels was stained with Coomassie brilliant blue R-250, (0.25% w/v Coomassie brilliant blue R-250 in 50% v/v methanol and 10% v/v acetic acid in distilled water), while the second gel was used for immunoblotting.

The reproducibility of the electrophoresis procedure was monitored by running protein extracts of all isolates on three separate gels with the reference samples and molecular weight markers (BioRad, prestained Precision Plus molecular weight markers) on each of the slab gels under identical conditions.

Total protein profiles analysis

Coomassie-stained gels were scanned with the Odyssey infrared imaging system (Li-Cor, Lincoln, NE, USA) and the images stored as TIFF files. Total protein profiles were analysed using the BIONUMERICS version 3.0 software package (Applied Maths, Sint-Martens-Latem, Belgium). Following conversion, normalization, and background subtraction with mathematical algorithms, levels of similarity between fingerprints were calculated with the Pearson product–moment correlation coefficient (*r*). Cluster analysis was performed with the unweighted pair-group method using average linkages (UPGMA) (vanOoyen 2001).

Immunoblotting

Unstained gels containing the resolved *Edwardsiella* proteins were electroblotted onto nitrocellulose membranes according to the method of Towbin, Staehelin and Gordon (1979), using a Criterion (Bio-Rad) electroblotting unit with a setting of 100 V for 30 min. Following transfer of proteins, the nitrocellulose membranes were equilibrated for 30 min in Starting-Block (Pierce Biotechnology) blocking buffer. The blots were rinsed and immersed in appropriate dilutions of anti-*E. ictaluri* (AL-93-58) or anti-*E. tarda* (AL-98-87) polyclonal goat antibody or monoclonal antibody (MAb AA224,) specific for *E. ictaluri* (Klesius & Horst 1991) and allowed to react for 60 min at room temperature. Affinity-purified, horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin (Jackson ImmunoResearch, West Grove, PA, USA) diluted 1:5000 was used as second antibody for probing blots treated with mouse MAb AA224 and affinity-purified, horseradish peroxidase-conjugated rabbit anti-goat immunoglobulin (Jackson ImmunoResearch) diluted 1:5000 was used on blots treated with polyclonal goat anti-*E. ictaluri* or *E. tarda* antibodies. Specific antibody-bound epitopes were detected following treatment of blots with the chromogenic substrate 4-chloro-1-naphthol (BioRad). Blots containing *E.ictaluri* and/or *E. tarda* were similarly probed with serum from an unimmunized goat as controls.

Bacterial fatty acid analysis

Preparation of fatty acid methyl esters (FAMEs) from bacteria grown at 28 °C on sheep blood agar plates

was done according to the Microbial Identifications Systems (MIS) (MIDI, Newark, DE, USA) version 4.5 procedure. Briefly, bacteria were saponified with NaOH (15% w/v) in aqueous methanol (50% v/v), methylated with methanolic HCl, extracted in hexane/methyl-tert-butyl ether (1:1), followed by washing in 3 mL of base wash (10.8 g NaOH, 900 mL deionized distilled water). The upper organic phase was removed and placed in gas chromatography vials for analysis. An Agilent 6850 (Agilent Technologies, Palo Alto, CA, USA) gas chromatographic system equipped with split injector, flame ionization detector and a fused silica column (30 m × 0.2 mm × 0.33 mm) was used with hydrogen as the carrier gas. The operating system was Chemstation (Agilent) and Sherlock MIS (MIDI). The temperature program ramped from 170 °C to 288 °C at 28 °C min^{-1} with a split ratio of 40:1 and head pressure of 144.8 kPa. Fatty acid methyl esters analysis was conducted on three sets of each isolate cultured and treated under identical conditions. Fatty acid methyl esters data were imported into BioNumerics to calculate the similarity levels between isolates using the Pearson product-moment correlation coefficient. UPGMA was used to generate the corresponding dendrogram.

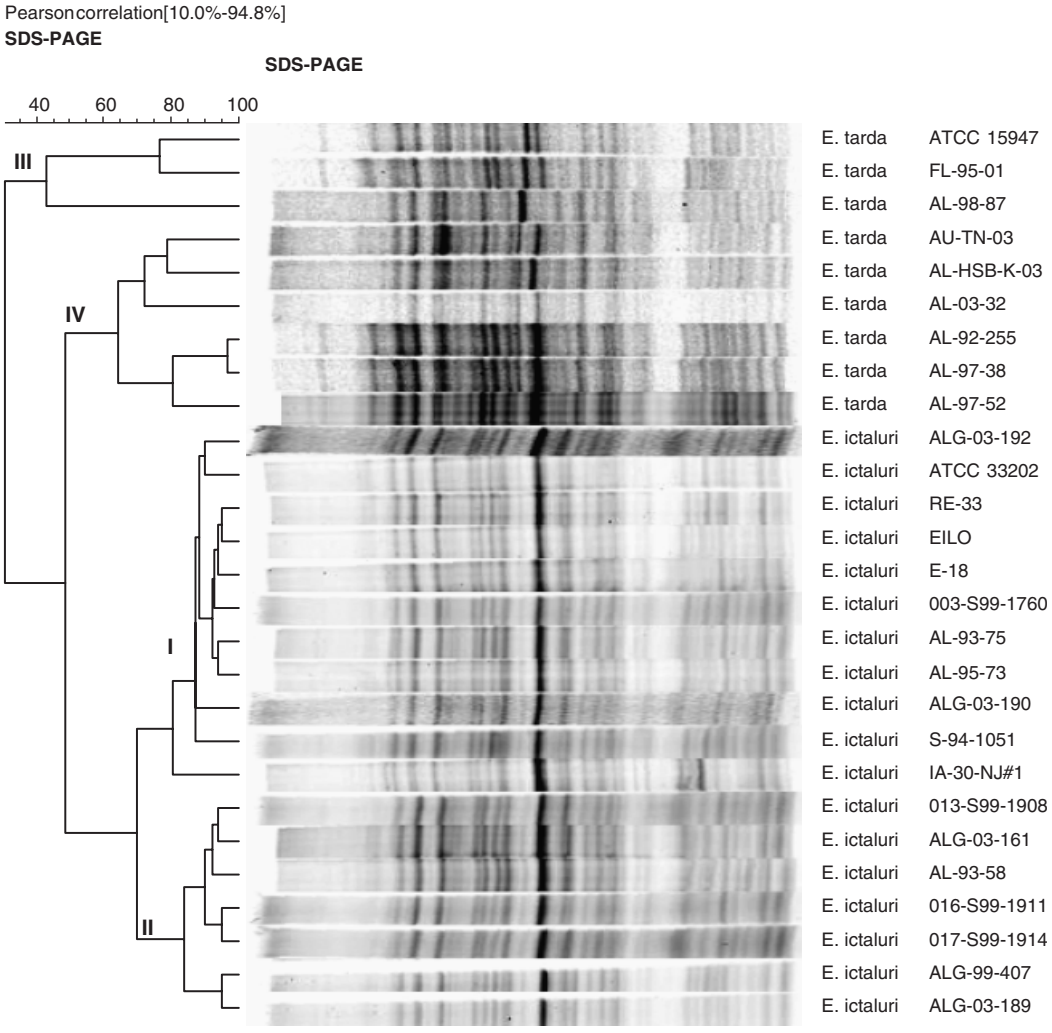

Figure 1 Total protein profiles of all isolates analysed in the study. The dendrogram was derived by unweighted pair-group method using average linkages (UPGMA) cluster analysis of the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) whole-cell protein profiles of 18 *Edwardsiella ictaluri* and nine *E. tarda* isolates. The tracks show the processed band patterns after conversion, normalization, and background subtraction. Levels of linkage are expressed as the Pearson product–moment similarity coefficient. Defined clusters are numbered as I, II, III and IV.

Results

Biochemical reactions

We used the distinctive biochemical traits of these two species (*E. ictaluri* and *E. tarda*) to establish the identities of the isolates. Biochemical tests revealed that all *E. ictaluri*, and *E. tarda* isolates conformed to the typical reaction profiles established for the respective species (Farmer 2003). All isolates of *E. ictaluri* and *E. tarda* fermented glucose, maltose and mannose and caused deamination of phenylalanine, but were differentiated on the basis of indole production, methyl red reduction and hydrogen sulphide production. The latter three criteria produced a positive reaction with *E. tarda* and a negative result with *E. ictaluri*. Randomly picked isolates tested more than twice, consistently yielded identical results.

Total protein profiles analysis

One-dimensional SDS-PAGE of whole-cell protein extracts of the 18 *E. ictaluri* and nine *E. tarda* isolates revealed protein profiles containing 32–35 discrete bands with molecular weights ranging within 10–250 kDa. The dendrogram derived following numerical analysis of the protein electrophoregrams is shown in Fig. 1. As was expected, *E. ictaluri* displayed a lower intraspecific variability than *Edwardsiella tarda*. Although two main clusters (I, II) could be defined within the species, all *E. ictaluri* isolates clustered together at 70% similarity or higher. Isolates did not reveal any distinctive clustering correlated with geographic distribution and the single isolate from the tadpole madtom (*Noturus gyrinus*, Mitchill) showed a banding pattern appreciably similar to the

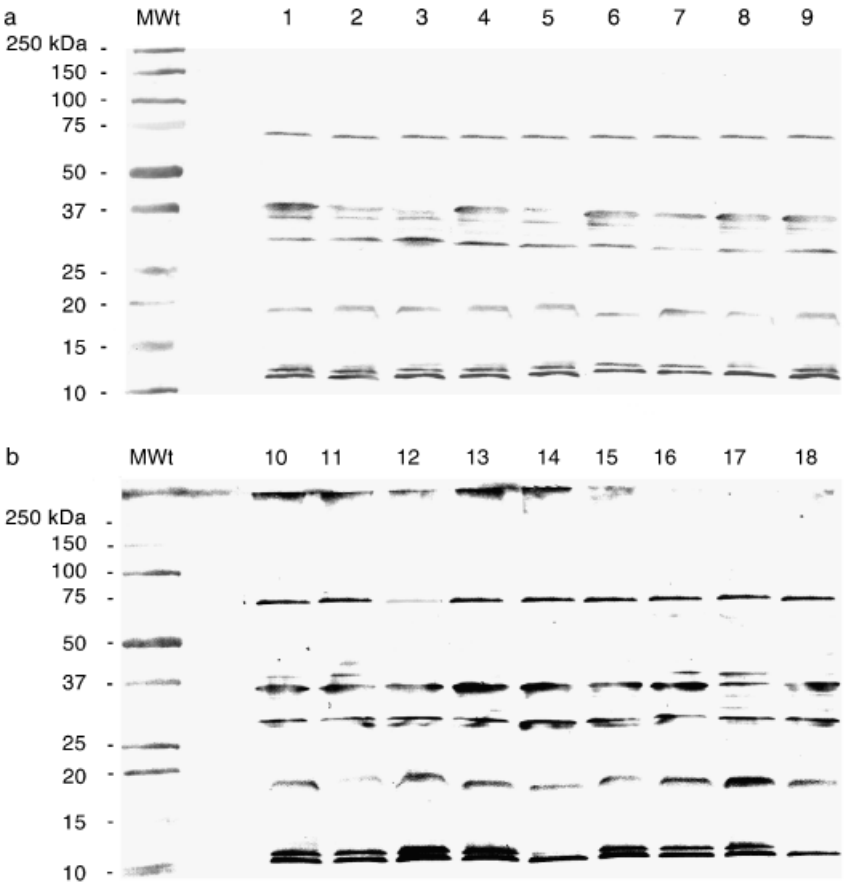

Figure 2 (a and b) Immunoblots of *Edwardsiella ictaluri* probed with polyclonal goat anti-*E.ictaluri* serum. Isolates arranged in lanes: 1, 016-S99-1911; 2, 017-S99-1914; 3, 013-S99-1908; 4, 003-S99-1760; 5, S-94-1051; 6, ALG-03-189; 7, ALG-03-190; 8, ALG-99-407; 9, ALG-03-192; 10, ALG-03-161; 11, AL-93-75; 12, AL-93-58; 13, AL-95-73; 14, RE-33; 15, E-18; 16, EILO; 17, IA-30-NJ#1; 18, ATCC-33202. Antigens at apparent molecular mass positions (from top to bottom) of 70, 37, 30, 18 and 12 kDa were recognized in all isolates of *E. ictaluri*. At 37 and 18 kDa, respectively, a duplet of bands were apparent. Recombinant Precision Plus (BioRad) prestained molecular weight markers are shown in lane marked MWt.

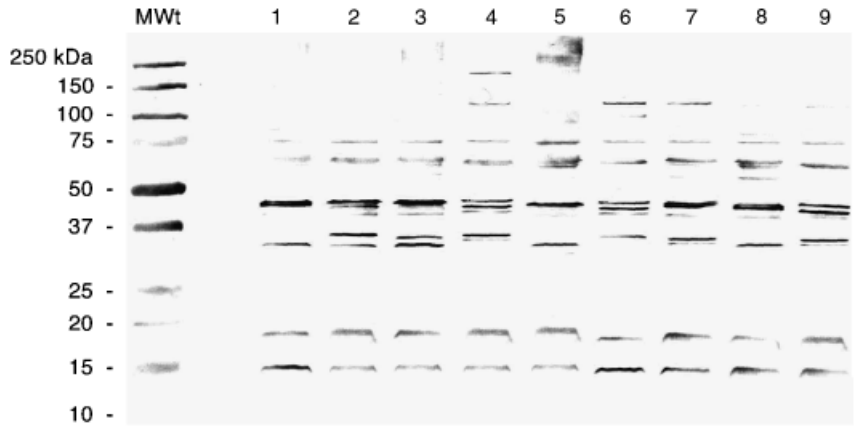

Figure 3 Immunoblot of *Edwardsiella tarda* probed with polyclonal goat anti-*E.tarda* serum. Isolates arranged in lanes: 1, AU-TN-03; 2, AL-HSB-K-03; 3, AL-03-32; 4, AL-98-87; 5, AL-97-052; 6, ATCC-15947; 7, AL-92-255; 8, AL-97-38; 9, FL-95-01. Antigens at apparent molecular mass positions (from top to bottom) of 71, 66, 46, 37, 18 and 15 kDa were recognized in all *E. tarda* isolates. In some isolates, the bands at 46 and 37 kDa, respectively, appeared as duplets or triplets. Additional antigenic epitopes were apparent in lanes 4, 6, 7 and 8. Molecular weight markers are shown in the lane marked MWt.

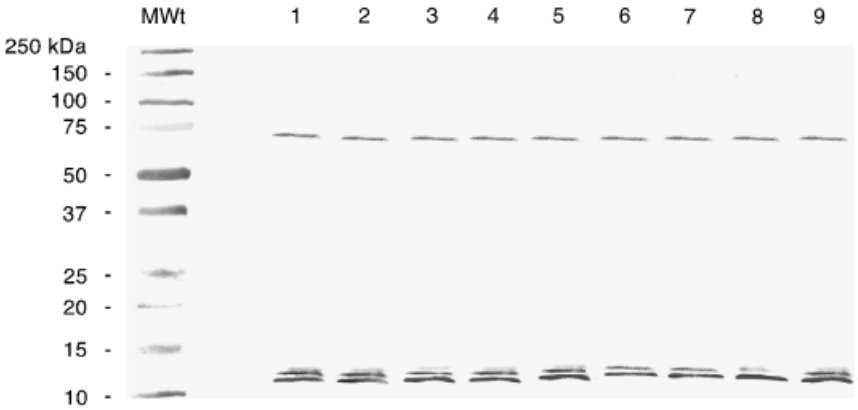

Figure 4 Immunoblot of *Edwardsiella ictaluri* isolates arranged in the order as in Fig. 2a and probed with MAb AA224. Antigenic epitopes are at apparent molecular mass positions of 71 and 12 kDa respectively. Antigens at 12 kDa appeared as a duplet of bands. Molecular weight markers are shown in the lane marked MWt. (*Note*: As the banding pattern with MAb AA224 was similar in all 18 isolates of *E. ictaluri*, only one of two blots are shown).

profiles presented by other *E. ictaluri* isolates from catfish (*Ictalurus punctatus*) except for a single low molecular weight band. *E. tarda* isolates revealed a higher degree of diversity showing as low as 30% similarity. The species was also split into two main clusters (III, IV). Cluster IV was more similar to *E. ictaluri* than to the other *E. tarda* isolates joining clusters I and II at 50% similarity. Cluster III, which includes the *E. tarda* type strain (ATCC-15947, a human isolate) and two other isolates from catfish shared only 30% similarity with the other *Edwardsiella* isolates. Overall, the analysis of the total protein profiles confirmed a higher homology level within *E. ictaluri*, while *E. tarda* displayed a higher degree of intrinsic diversity. Some *E. tarda* isolates had profiles more in common with *E. ictaluri* than with other *E. tarda* isolates.

Immunoblotting

Immunoblots showing the antigenic profile of 18 isolates of *E. ictaluri* (Fig. 2a and b) and nine of *E. tarda* isolates (Fig. 3) are shown. The major antigenic epitopes of *E. ictaluri* were in the approximate molecular mass ranges of 12, 18, 30, 37 and 70 kDa (Fig. 2a and b). The epitopes recognized by polyclonal goat antiserum against *E. ictaluri* had an identical molecular

mass distribution across all 18 isolates. The same was true when the Western blots of *E. ictaluri* were probed with the MAb AA224 (Fig. 4). However, only epitopes at two molecular mass positions were recognized with MAb AA224. An upper band with an apparent molecular mass of 70 kDa and a duplet of bands at an approximate position of 12 kDa. There was considerable variation in the epitopes recognized by polyclonal goat antiserum among the *E. tarda* isolates (Fig. 3). The major bands were located at apparent molecular mass positions of 15, 18, 37, 46, 66 and 71 kDa. In some of the isolates, the bands appeared as closely spaced repeating duplets or triplets and isolates 4, 6, 7 and 8 had additional antigenic bands (Fig. 3). No MAbs were available for probing the antigens of *E. tarda* isolates.

FAME composition

The mean percent fatty acid composition of all isolates of both *Edwardsiella* species is given in Table 2. Descriptive nomenclature of the major FAMEs identified is presented in Table 3. Thirteen FAMEs that were detected at a level greater than 1% belonged to the categories of saturated, unsaturated and cyclopropane fatty acids respectively (Table 3). Some FAMEs, as for

Table 2 Mean percent fatty acid composition for all isolates tested

Fatty acid methyl esters (FAME)											
Bacteria	**14:0***	**14:502 unknown**	**16:0**	**17:0Δ**	**18:0**	**18:1ω7cis**	**18:1ω9cis**	**19:0Δω8cis**	**Summed† feature 2**	**Summed feature 3**	**Summed feature 6**
E. ictaluri	8.02	1.44	25.79	4.35	1.32	11.94	1.31	2.45	9.42	27.68	1.22
	(1.76)	(0..58)	(7. 03)	(2. 08)	(0. 65)	(7. 64)	(0.80)	(1.76)	(0. 97)	(2.76)	(0.49)
E. tarda	10.69	1.23	29.42	12.38	1.07	6.71	1.14	1.40	8.51	15.28	1.39
	(1.34)	(0.77)	(2.20)	(2.79)	(0.36)	(2.30)	(0.56)	(1.04)	(1.02)	(4.49)	(0.63)

*Numbers before the colon indicate the number of carbon atoms and the numbers after the colon refer to the number of double bonds. ω7cis, ω8cis, and ω9cis indicate cis isomers with double-bond positions from hydrocarbon end of carbon chain; Δ indicates the presence of a cyclopropane ring in the carbon chain. Trace amounts (< 1%) of FAME recorded are not shown. Numbers within parentheses denote the standard deviation.

†Summed feature (SF) denotes two peaks exhibiting overlapping retention times with fatty acids in each of two elution profiles.

E. ictaluri, Edwardsiella ictaluri; E. tarda, Edwardsiella tarda.

Table 3 Nomenclature of fatty acid methyl esters of *Edwardsiella ictaluri* and *E. tarda*

Shorthand name		Systematic name		Trivial name
Saturated fatty acids				
14:0	–	Tetradecanoic acid	–	Myristic acid
16:0	–	Hexadecanoic acid	–	Palmitic acid
18:0	–	Octadecanoic acid	–	Stearic acid
Unsaturated fatty acids				
18:1 ω7*cis*	–	*cis*-7-Octadecenoic acid	–	Unknown
18:1 ω9*cis*	–	*cis*-9-Octadecenoic acid	–	Oleic acid
Cyclopropane fatty acids				
17:0Δ	–	*cis*-9,10-Methylene hexadecanoic acid	–	Analog of margaric acid
19:0Δω8cis	–	*cis*-8-Methylene Octadecanoic acid	–	Analog of Lactobacillic acid
SF*				
SF-2 = 14:0 3-OH	–	3-Hydroxytetradecanoic acid	–	3-Hydroxymyristic acid
16:1 iso I	–	Hexadecenoic acid, isomer I	–	Unknown
SF-3 = 15:0 iso 2-OH	–	2-Hydroxypentadecanoic acid	–	Unknown
16:1 cis 7	–	*cis*-7-Hexadecenoic acid	–	Palmitoleic acid
SF-6 = 18:0 anteiso	–	Anteisononadecanoic acid	–	Unknown
18:2 *cis*6,*cis*9	–	Octadecatrienoic acid	–	Linolenic acid

*Summed feature (SF) denotes two peaks exhibiting overlapping retention times with fatty acids in each of two elution profiles. Fatty acids of SF-2,3 and 6 as presented in MIDI version 4. Nomenclature according to Komagata and Suzuki (1987); Stead (1992).

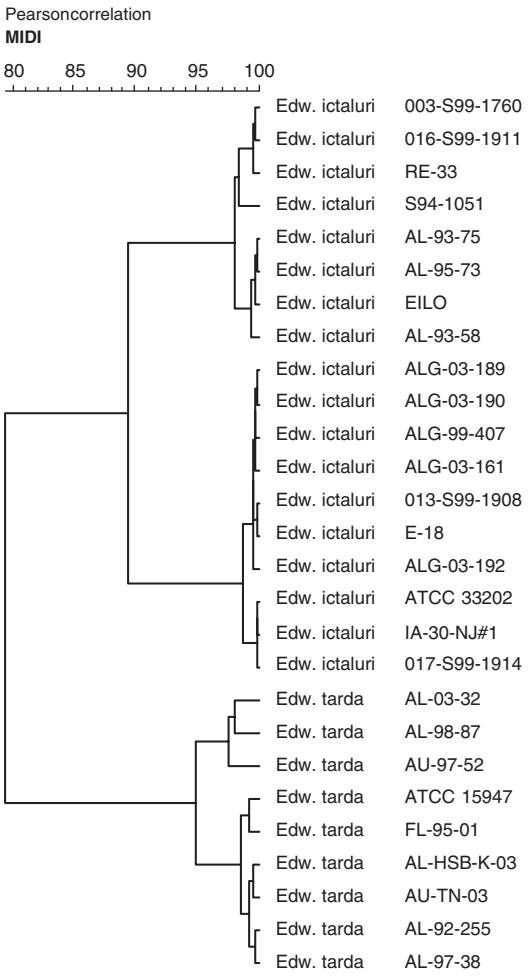

Figure 5 Dendrogram based on Pearson product–moment correlation analysis of fatty acid profiles of *Edwardsiella ictaluri* and *E. tarda* isolates. Numbers on the horizontal axis indicate the percentage similarity.

example the 15:0 (pentadecanoic acid), occurred in trace amounts (<1%) and were not included. The 16:0 (hexadecanoic acid) and the unsaturated forms of 16 and 18 carbon atoms were the most abundant species of FAMEs in both *E. ictaluri* and *E. tarda*. All of the unsaturated fatty acids were *cis* isomers. There were no major qualitative differences between the profiles; however, the *E. tarda* FAME profiles differed from those of *E. ictaluri* quantitatively (Table 2). The concentration of 14:0 (tetradecanoic acid) and 17:0Δ(*cis*-9,10-methylene hexadecanoic acid) with 10.69% and 12.38%, respectively, were appreciably greater in *E. tarda* compared with 8.02% and 4.35% respectively in *E. ictaluri*. Contrariwise, *E. ictaluri* showed an abundance (11.94%) of the 18:1 ω7*cis* (*cis*-7-octadecenoic acid) compared with *E. tarda* (6.71%). A substantial difference was also seen in the summed feature 3 (SF-3) between the two species of *Edwardsiella* (Table 2). A dendrogram reflecting the levels of similarity among the isolates is presented in Fig. 5. By using FAME analysis, both species could be clearly discriminated. All *E. ictaluri* isolates clustered together at 95% similarity. Although the dendrogram shows two distinct groups within the species, the cut-off value for this technique is reported to be around 90% similarity (Shoemaker, Arias, Klesius & Welker, 2005). Therefore, no true groups with a similarity over 90% could be inferred. All *E. tarda* isolates grouped together at a high similarity level (over 95%) forming a tight cluster. Both species cluster together at 80% similarity.

Discussion

Adaptive pleiotropic alterations occur among bacteria to cope with selective pressures exerted by the environment such as the host's immune response, altered tropism for a specific habitat niche driven by nutritional requirements or to extend the adaptable host range (Rainey, Moxon & Thompson 1993; Moxon, Rainey, Nowak & Lenski 1994; Foster 2000). Because of the high degree of bacterial plasticity, these changes may occur stochastically or because of programmed genomic rearrangements (Simon & Herskowitz 1985; Dobrindt & Hacker 2001). A wide variety of genotypic and phenotypic parameters have been used for detection and characterization of adaptive variations that occur within a given bacterial species. Because phenotypic manifestations are largely a reflection of the intrinsic genotypic makeup in bacteria, a high degree of congruence has been observed between the molecular genetic and associated phenotypic characteristics (Owen & Jackman 1982; Costas 1992; Goodfellow & O'Donnel 1993; Palys, Berger, Mitrica, Nakamura & Cohan 2000; Cohan 2002). In the present study, we used three phenotypic criteria (electrophoretic protein profiles, antigenic epitopes, and cellular FAME composition) to distinguish putative variations among 18 isolates of *E. ictaluri* and nine isolates of *E. tarda* from different outbreaks of enteric septicaemic disease in fish. Initially all isolates were characterized biochemically and found to conform to the descriptive pattern characteristic for the respective type species of *Edwardsiella* in the family *Enterobacteriaceae* (Holmes *et al.* 1986; Farmer 2003). No biochemical test variation was found within isolates belonging to the same species.

Based on the numerical analysis of the SDS-PAGE protein patterns, *E. ictaluri* isolates could be divided into two distinct groups. However, they all clustered together at high percent (70%) of similarity. Visual examination of Coomassi-stained, SDS-PAGE profiles of whole-cell protein have revealed approximately 38 bands in *E. ictaluri* (Baldwin, Collins & Newton 1997). Our findings are in close agreement, in that both *E. ictaluri* and *E. tarda* revealed 32–35 protein bands by densitometric scanning. Upon analysis of the protein profiles of 14 *E. ictaluri* isolates by SDS-PAGE, and immunoblotting, Plumb and Klesius (1988) concluded that *E. ictaluri* are comprised of a homogeneous group. Newton *et al.* (1990) examined the outer membrane protein (OMP)-enriched fractions of 33 isolates of *E. ictaluri* from several species of fish, of different geographic locations and found that except for isolates from a green knife fish (*Eigemannia virescens*) and a white catfish (*Ictalurus catus*), the remaining isolates had identical OMP profiles. Notwithstanding, in the present study, isolates of *E. tarda* were distributed among two clusters grouping as low as 30% similarity. Based on our SDS-PAGE analysis, *E. tarda* is comprised of a relatively heterogeneous group compared with *E. ictaluri*. Analysis of OMP profiles of 27 *E. tarda* strains by Huang and Lu (2001) and 10 isolates of *E. tarda* by Darwish, Newton and Plumb (2001) have led to the conclusion that *E. tarda* is relatively heterogeneous. Recent proteomic analysis of extracellular proteins (Tan *et al.* 2002) and virulence determinants of *E. tarda* (Srinivas Rao, Yamada, Tan & Leung 2004) have lent to a similar conclusion.

Antigenically, *E. ictaluri* has been observed to be considerably homogeneous (Plumb & Klesius 1988). Our studies corroborate these findings. We observed five antigenic bands with immune sera raised in goats and these occurred in *E. ictaluri* at apparent molecular mass positions of 12, 18, 30, 37 and 70 kDa respectively. It is possible that the prominant antigens at 34 and 60 kDa identified with immune catfish serum by Plumb and Klesius (1988) correspond with the 37 and 70 kDa bands detected by us and that the 39 kDa band observed by Baldwin *et al.* (1997) correspond to the 37 kDa antigen detected in the present study. As fish have only one class of immunoglobulin (i.e., IgM), it is plausible that only a limited number of epitopes are detected with catfish antibodies compared with the polyclonal goat antiserum to *E. ictaluri* and *E. tarda* used in the present study. Contrary to the remarkable homogeneity seen in the antigenic profiles of *E. ictaluri*, considerable variation in both the molecular mass position and intensity was seen in the *E. tarda* blots probed with polyclonal goat anti-*E. tarda* serum. Kawai, Liu, Ohnishi and Oshima (2004), using polyclonal rabbit antiserum against formalin killed cells of *E. tarda* strain EF-1, characterized a prominent 37 kDa OMP that was conserved in six different serotype strains of *E. tarda*. However, they (Kawai *et al.* 2004) did not mention the recognition of any additional antigens that may have been present. In addition to a prominent 37 kDa epitope, we observed five other epitopes that were present in variable permutations among all isolates *E. tarda* examined.

All of the isolates of both *E. ictaluri* and *E. tarda* included in this study revealed methyl esters of fatty acids containing from 14 to 19 carbon atoms in whole bacterial cell extracts. The most abundant fatty acids 16:0 (tetradecanoic acid), 16:1 *cis* (hexadecenoic acid) and 18:1 *cis* (octadecanoic acid) are known to be the major inner membrane constituents present in the profiles of most if not all Gram-negative bacteria (Bøe and Gjerde 1980; Komagata & Suzuki 1987; Welch 1991; Stead 1992). Cyclopropane and hydroxylated fatty acids were also present in both *E. ictaluri* and *E. tarda* and these fatty acids are known to be present in other members of the *Enterobacteriaceae* family (Grogan & Cronan Jr 1997). Interestingly, by using FAME analysis, *E. tarda* appeared as a more homogeneus species than *E. ictaluri* as all *E. tarda* isolates clustered at 96% similarity versus 90% for *E. ictaluri*. However, previous data (Shoemaker *et al.*, 2005) obtained in our lab, suggest that taking into account the reproducibility and the error introduced when analysing the FAME profiles, differences of similarity over 95% should not be considered.

Our finding on the FAMEs of *E. ictaluri*, conforms to the inference of a previous study by Klesius, Lovy, Evans, Washuta and Arias (2003), who observed a high degree of homogeneity (at $r = 85\%$) among 11 isolates of *E. ictaluri* by cluster analysis of BIOLOG and FAME profiles. The results of the present study share considerable similarity with the species of FAMEs found in other known fish-pathogenic Gram-negative bacteria (Romalde, Margarinos, Turnbull, Baya, Barja & Toranzo 1995) and also show a high degree of relatedness between the *E. ictaluri* and *E. tarda* isolates.

We recently examined the genetic relatedness of *E. ictaluri* and *E. tarda* isolates, including 17 of *E. ictaluri* isolates and three of the *E. tarda* isolates examined phenotypically in the present study, by comparing the sequence of the 16S–23S intragenic spacer

regions (ISRs) (Panangala, van Santen, Shoemaker & Klesius 2005). All the *E. ictaluri* isolates had identical ISR sequences, while the small sample of *E. tarda* isolates displayed two distinct ISR sequences, mirroring the relative homogeneity of the *E. ictaluri* isolates and the heterogeneity of the *E. tarda* isolates demonstrated by the phenotypic analysis. Analysis of the 16S–23S intergenic spacer regions of the rRNA operons in *E. ictaluri* and *E. tarda* revealed two groups of *E. tarda* (Panangala *et al.* 2005). However, the groups generated by the different phenotypic and genotypic analysis of *E. tarda* do not exactly coincide.

Bringing into focus the many reasons advanced for clonality in studies of bacterial population genetics (Spratt & Maiden 1999; Feil & Spratt 2001; Cohan 2002; Spratt 2004), we suggest that *E. ictaluri* appears to be adapted to a clonal existence wherein a complete, albeit a minimal, ensemble of archived genes (core gene pool), proteins and antigenic determinants are maintained. There appears to be some operational selective advantage for conservation of intrinsic traits. Many studies imply that highly successful clonal populations exhibit linkage disequilibrium, whereas by contrast, linkage equilibrium is characteristic of populations where mutations occur frequently and in random assortment (Tibayrenc 1995; Spratt & Maiden 1999; Bart, Barnabe, Achtman, Dankert, van der Ende & Tibayrenc 2001). Clonal bacterial populations are therefore characterized by lower levels of genetic and phenotypic diversity (Levin 1981; Bart *et al.* 2001). It is plausible that the restricted host range seen with *E. ictaluri* likely accounts for its adaptive genotype to selectively survive within a favorable eco-niche and hence the uniquely monomorphic characteristics. In contrast, *E. tarda* appears to be more plastic, phenotypically more polymorphic and capable of adapting to survive within a broad host range. Understanding the phenotypic characteristics among isolates of a given pathogen is important for detecting the emergence of new and/or more virulent isolates, understanding the epidemic spread of microbes and for formulating effective strategies for immunization and preventing the spread of disease.

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